Journal of Chromatography B, 963 (2014) 106–112

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Urine homogentisic acid and tyrosine: Simultaneous analysis by liquid chromatography tandem mass spectrometry

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ARTICLE INFO

Article history:
Received 28 March 2014
Accepted 1 June 2014
Available online 7 June 2014

Keywords:
Alkaptonuria
Homogentisic acid
Tyrosine
Liquid chromatography
Tandem mass spectrometry

ABSTRACT

Alkaptonuria (AKU) is a rare debilitating autosomal recessive disorder of tyrosine metabolism. Deficiency of homogentisate 1,2-dioxygenase results in increased homogentisic acid (HGA) which although excreted in gram quantities in the urine, is deposited as an ochronotic pigment in connective tissues, especially cartilage. Ochronosis leads to a severe, early-onset form of osteoarthritis, increased renal and prostatic stone formation and hardening of heart vessels. Treatment with the orphan drug, Nitisinone, an inhibitor of the enzyme 4-hydroxyphenylpyruvate dioxygenase has been shown to reduce urinary excretion of HGA, resulting in accumulation of the upstream pre-cursor, tyrosine. Using reverse phase LC–MS/MS, a method has been developed to simultaneously quantify urinary HGA and tyrosine. Using matrix-matched calibration standards, two product ion transitions were identified for each compound and their appropriate isotopically labelled internal standards. Validation was performed across the AKU and post-treatment concentrations expected. Intrabatch accuracy for acidified urine was 96–109% for tyrosine and 94–107% for HGA; interbatch accuracy (n = 20 across ten assays) was 95–110% for tyrosine and 91–109% for HGA. Precision, both intra- and interbatch was <10% for tyrosine and <5% for HGA. Matrix effects observed with acidified urine (12% decrease, CV 5.6%) were normalised by the internal standard. Tyrosine and HGA were proved stable under various storage conditions and no carryover, was observed. Overall the method developed and validated shows good precision, accuracy and linearity appropriate for the monitoring of patients with AKU, pre and post-nitisinone therapy

1. Introduction

Alkaptonuria (AKU) was the first inborn error of metabolism described by Garrod, in 1902 [1] (OMIM # 203500). It is a rare autosomal recessive disorder, which results from a deficiency in homogentisate 1,2-dioxygenase activity in the liver [2,3] (Fig. 1). In the absence of the enzyme, homogentisic acid (HGA) is excreted in the urine in gram quantities [4–6] (equivalent to mmol/L concentrations), which turns black upon standing or alkalisation. HGA circulates at lower concentrations in plasma (μmol/L) and is oxidised to benzoquinones which polymerise and bind to proteins, particularly in connective tissues including cartilage [7–11]. This process leads to ochronosis – a blue-black discolouration of the connective tissues especially cartilage. The mechanism of this remains unclear. Ochronotic pigmentation causes early onset degenerative arthritis of the spine and large weight bearing joints leading to increased pain and premature joint replacement. Aortic stenosis has been described as a cardiac complication of the ochronosis process [12,13] and in severe cases has led to aortic valve replacement. Additionally, an increased incidence of kidney stone formation is reported with an increase in prostate stones in males [5,8]. The formation of benzoquinones results in additional formation of reactive oxygen species and free radicals, which are suggested to play a significant role in the aetiology of AKU arthritis [14].

Although current therapy for AKU is palliative, there have been several reported trials of Nitisinone (2-(2-nitro-4-fluromethylbenzoyl)-1,3-cyclohexanedione [5,6,9,15,16]. Nitisinone is a potent inhibitor of the second enzyme in the tyrosine pathway, p-hydroxyphenylpyruvic acid oxygenase which has been shown to

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http://dx.doi.org/10.1016/j.jchromb.2014.06.002
1570-0232/

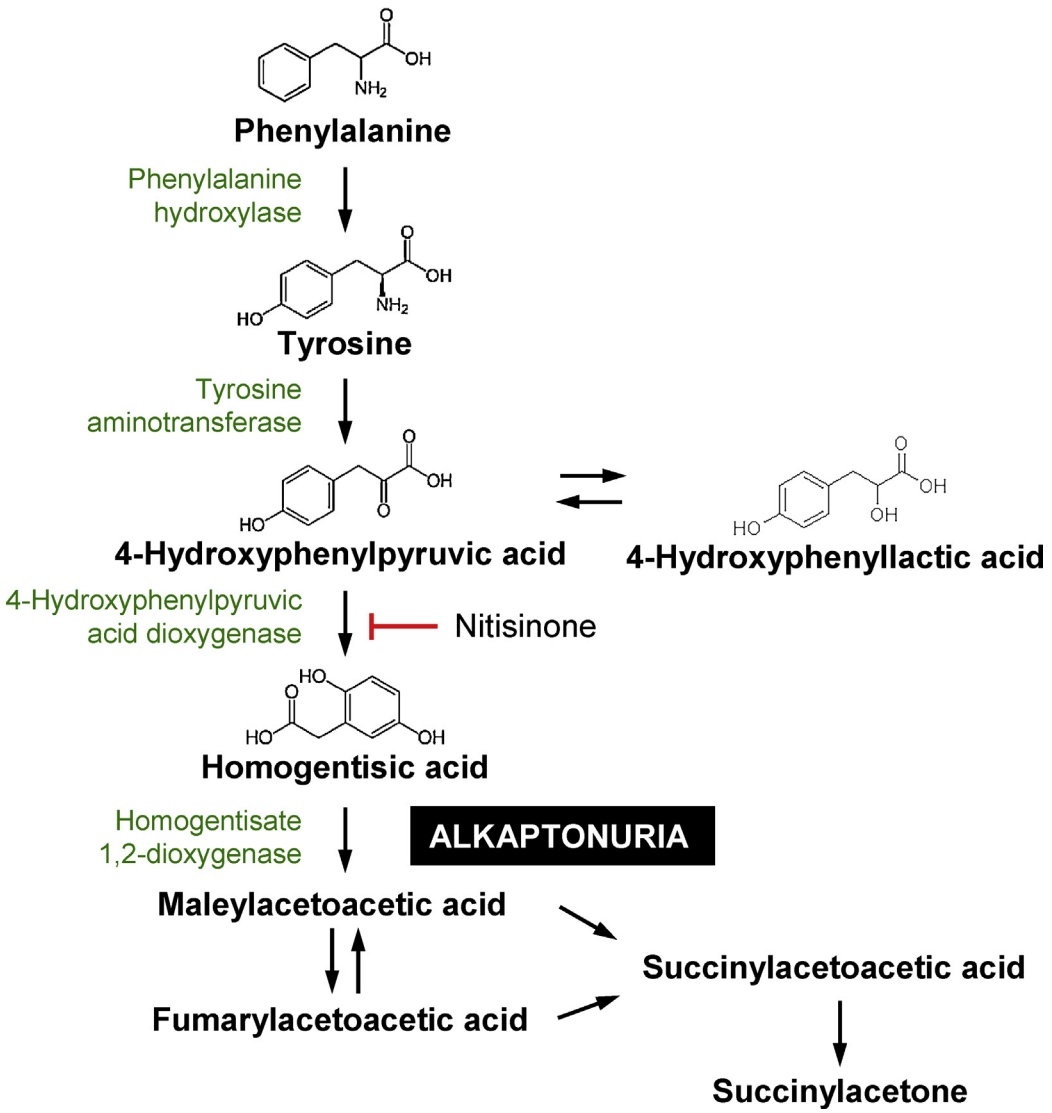

Fig. 1. Tyrosine degradation pathway showing the enzyme defect in alkaptonuria. Nitisinone blocks the enzyme prior to HGA.

lower circulating HGA and completely prevent pigmentation in AKU mice [17]. Nitisinone is approved for treatment of hereditary tyrosinaemia (OMIM #276700). It is currently being used at the National Alkaptonuria Centre (Royal Liverpool University Hospital, UK), in a large multi-centre clinical trial (DevelopAKUre Clinical Trials – www.akusociety.org) to evaluate its effectiveness in the treatment of AKU.

Currently published methods for quantitative and semi-quantitative analysis of HGA employ spectrophotometry, enzymatic spectrophotometry, gas chromatography mass spectrometry (GC–MS) and high performance liquid chromatography (HPLC) and integrated with mass spectrometry LC–MS/MS [17–23]. Urine tyrosine is generally measured as part of an amino acid screen with semi-quantitative reporting as part of a total screen, or quantitative analysis in select panels [24].

The diagnosis of AKU relies on clinical presentation and evaluation and quantitative or semi-quantitative analysis of urine HGA. Due to the instability of HGA in an alkaline environment, the preferred sample collection criterion is acidified urine. The aim of this study was to develop a quantitative method for the simultaneous measurement of urinary tyrosine and HGA using LC–MS/MS. For validation and comparison purposes, the method has been validated in both acidified and non-acidified urine matrices.

2. Materials and methods

2.1. Chemicals and materials

Tyrosine, tyrosine isotope-labelled internal standard (d_2-tyrosine) and HGA were obtained from Sigma–Aldrich UK. HGA isotope-labelled internal standard, $^{13}C_6$-HGA was obtained from Larodan Fine Chemicals (Sweden). LC–MS grade methanol and acetonitrile were obtained from Sigma Aldrich, UK. Formic acid was obtained from Biosolve. Water was purified in-house by DIRECT-Q 3UV Millipore water purification system. All dilutions and sample preparation was performed in glass. Oxygen free nitrogen was supplied by a Peak nitrogen generator.

2.2. Instrumentation and operating conditions

All analysis were performed on an Agilent 6490 Triple Quadrupole mass spectrometer with Jet-Stream® electrospray ionisation (ESI–MS/MS) coupled with an Agilent 1290 infinity UHPLC pump and HTC autosampler. All data processing both qualitative and quantitative analysis was performed using Mass Hunter software package.